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Allosteric Controls of Amphibolic Pathways in Bacteria

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INTRODUCTION

Traditionally, pathways of metabolism have been classified into two major categories, anabolic (or biosynthetic) and catabolic (or degradative). Davis (21) pointed out, however, that this classification leaves much to be desired. If one considers the classical example of a catabolic pathway (namely, glycolysis), the difficulty of nomenclature is obvious. Bacteria growing on glucose as a carbon source utilize glycolytic enzymes as a catabolic pathway, but when growing on a C_3 or C_4 organic acid basically the same set of enzymes function as an anabolic pathway. To circumvent these ambiguities of classification, Davis (21) coined the term *amphibolic* to designate pathways that fulfill both an anabolic and a catabolic function. As understood at the present time, amphibolic pathways encompass the enzymes of glycolysis, the hexose monophosphate pathway, glucogenesis, and the tricarboxylic acid cycle. The importance of amphibolic pathways lies in the fact that they furnish carbon skeletons (e.g., oxalacetate, phosphoenolpyruvate, etc.) which are utilized not only for biosynthetic purposes but also for the generation of energy by their complete degradation through the terminal oxidative pathways. For the efficient operation of such metabolic routes, bacteria have evolved control mechanisms which are unique to the enzymes of carbohydrate metabolism and which ensure a correct, coordinated flow of carbon fragments into the biosynthetic channels and into the energy-generating pathways. The purpose of this review is not only to enumerate these enzymatic control mechanisms and to point out heuristic generalizations but also to examine critically the teleonomic significance of the occurrence of diverse types of control loops.

Since this review is primarily directed towards an examination of the enzymatic controls of bacterial metabolism, it is useful to point out at the very outset that controls of carbohydrate metabolism found in procaryotes tend to be quite different and more numerous than those found in eucaryotes. This is probably because bacteria lack the rigid, compartmentational controls such as are afforded to the eucaryotic cells by the presence of mitochondria. Because of these organelles, the glycolytic segment which supplies C_3 fragments is physically separated in eucaryotes from the terminal oxidative pathway, and this compartmentation determines to some extent the fate of the carbon skeleton. Compartmentation is not entirely absent, however, in all bacteria. Many gram-positive and gram-negative forms do have some semblance of an organized structure (mesosomes) partly comparable to mitochondria (24, 95, 96), and the association of some enzymes of the tricarboxylic acid cycle with the mesosomes

has been reported (78). For orientation regarding the controls of carbohydrate metabolism in eucaryotes, the reader is referred to an excellent review by Scrutton and Utter (111). In the following account, I have made no attempts to summarize information which deals with regulation of amphibolic pathways by genetic repression and derepression mechanisms, except when such information is incidental to and complements the discussion of allosteric controls.

NATURE OF ENZYMATIC CONTROLS

Enzyme Controls in Biosynthetic and Catabolic Pathways

For a better appreciation of the complexities of controls of amphibolic pathways, it is pertinent to discuss briefly the principles of control of the enzyme activities of strictly biosynthetic and catabolic pathways. Chronologically, the first clear-cut evidence for any direct control of the activity of an enzyme by metabolites came in 1956 from the study of the isoleucine-valine biosynthetic pathway (127) and the pyrimidine biosynthetic pathway in *Escherichia coli* (144). In a classic paper, Umbarger (127) demonstrated that isoleucine, the end product of threonine-isoleucine pathway, inhibited the first enzyme (threonine deaminase, EC 4.2.1.16) of its pathway. This he interpreted as a negative feedback mechanism. At the present time, a large number of enzymes are known which are susceptible to a negative feedback inhibition of their activity, and it is a fair generalization that in purely biosynthetic pathways the major control exerted is end product or negative feedback control (4, 114, 126, 128). Very sophisticated variations of this basic homeostatic mechanism have been evolved by various bacteria and other organisms. Witness, for example, the occurrence of cooperative feedback (12, 84), concerted feedback (20, 88), and cumulative feedback (140)—to name a few—in different biosynthetic pathways.

In direct contrast, however, strictly catabolic sequences do not generally seem to be regulated by end-product inhibition (40–42, 115). The strictly catabolic class of enzymes (21) would be those which convert diverse, nonessential carbon sources into the intermediates of amphibolic pathways (such as the tricarboxylic acid cycle). Numerous such pathways are known in bacteria (40, 42, 75). Degradation of aromatic rings, (+) camphor, arginine, branched-chain amino acids, purine and pyrimidine bases, and many other compounds, when used as sole carbon sources, is achieved largely by catabolic routes. In nearly all of these cases, the compound in question is broken down to an intermediate of the tricarboxylic acid cycle. A brief discussion of these pathways is given by Gibson (34). The activity of initiating enzymes of the degradative pathways is generally controlled by compounds which serve as indicators of the energy state of a cell such as inorganic phosphate (Pi), pyrophosphate, and adenine or other purine nucleotides. The biodegradative threonine deaminase of *E. coli* (129) and aspartase (EC 4.3.1.1) of *Enterobacter aerogenes* (136) and *Bacterium cadaveris* (137) are both activated by adenosine-5′-phosphate (AMP). The fermentation of threonine in *Clostridium tetanomorphum* is also linked to energy metabolism (82, 125, 135). Histidase (EC 4.3.1.3.), the first enzyme of the histidine degradative pathway in *Pseudomonas aeruginosa* (66), is inhibited by pyrophosphate, and this inhibition is relieved by AMP and guanosine-5′-diphosphate (GDP).

The examples given above serve to show that the biosynthetic and catabolic pathways differ mainly in the nature of the regulatory signal utilized for control. In the former, the signal is the specific end product of the pathway; in the latter, the signal is an ultimate product (or easily interconvertible products) of energy metabolism common to the diverse catabolic channels.

End-Product Control in Amphibolic Pathways

It has already been mentioned that amphibolic pathways serve a catabolic as well as an anabolic function. It is understandable, therefore, that the control characteristics specific for the catabolic pathways (feedback by energy indicators) and the biosynthetic sequences (negative feedback by end product) be both represented in amphibolic pathways. To appreciate this statement fully, the schematic diagram shown in Fig. 1 can be divided operationally into four linear segments:

(i) Fructose-6-phosphate → fructose diphosphate (FDP) → → phosphoenolpyruvate (PEP)
(ii) PEP → oxalacetate → aspartate (or malate)
(iii) Pyruvate → → → acetyl coenzyme A (CoA)
(iv) Acetyl-CoA → citrate → isocitrate → α-ketoglutarate (α-KG)

In each of these sequences, the last-named metabolite may be considered to be the end product of the pathway starting out from the first-named intermediate, and, in accord with the principles of end-product control (127), the last named compound causes a negative feedback inhibition of the first enzyme of its pathway. All of these enzymes seem to be allosteric, as defined by Monod, Changeux, and Jacob (80), and catalyze reactions which are physiologically irreversible. Blangy, Buc, and Monod (8), working with phosphofructokinase of *E. coli* (EC 2.7.1.11; first enzyme of

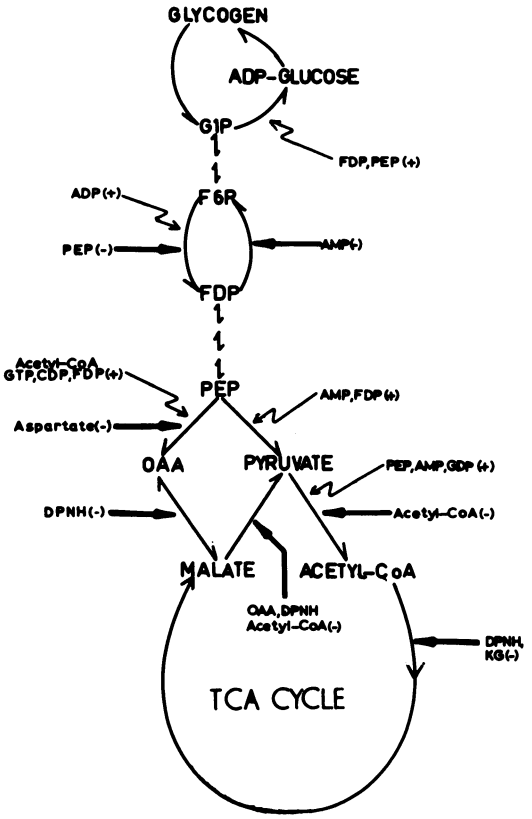

FIG. 1. *Schematic representation of some of the glycolytic, glycogenic, and terminal oxidation segments in E. coli and their control. Bold arrows indicate inhibition and the curved arrows denote activation. Plus and minus signs indicate activation or inhibition, respectively, by the various metabolites.*

sequence i), demonstrated that PEP inhibits the activity of this enzyme in an allosteric manner and that the inhibition is completely reversed by fructose-6-phosphate. Inhibition of phosphofructokinase by PEP was first demonstrated by Uyeda and Racker (130) in rabbit muscle preparations. In *Salmonella typhimurium* and *E. coli*, PEP carboxylase (EC 4.1.1.31), the first enzyme catalyzing the formation of oxalacetate, is inhibited by a number of dicarboxylic acids (18, 70, 85), of which aspartate is the most powerful inhibitor. In bacteria, as also in most other eucaryotes, three enzymes, PEP carboxylase (or, pyruvate carboxylase in some fungi, bacteria, and mammals), malic enzyme, and PEP carboxykinase are all theoretically capable of fixing carbon dioxide to produce oxalacetate. It was demonstrated in *S. typhimurium* (124) and in *E. coli* that only mutants which lack PEP carboxylase fail to grow on pyruvate or its precursors unless citric acid cycle intermediates are also added to the growth medium. Thus, it is clear that oxalacetate must only be produced in vivo by PEP carboxylase.

The third sequence, starting with pyruvate (to acetyl-CoA), is catalyzed by the well-known multienzyme complex (38, 59) constituted of pyruvate dehydrogenase (EC 1.2.4.1), dihydrolipoyl transacetylase (EC 2.3.1.12), and dihydrolipoyl dehydrogenase (EC 1.6.4.3.), which act sequentially to produce acetyl-CoA. Here again, acetyl-CoA exerts a negative feedback on the first enzyme (pyruvate dehydrogenase) of the complex in *E. coli* (110).

Finally, it has been shown (142) that α-KG, the end product of the sequence starting with acetyl-CoA, inhibits citrate synthetase (EC 4.1.3.7) of *E. coli* and *Salmonella* (133, 142) in an allosteric manner. While only segments of constitutive glycolytic channel and citric acid cycle are considered here, it should not be forgotten that new links to existing amphibolic sequences are created when bacteria grow on sources of carbon other than glucose. As examples, *E. coli* growing on glycerol dissimilates this compound through an inducible glycerol kinase (EC 2.7.1.30) and α-glycerophosphate dehydrogenase [EC 1.1.1.8 (39, 57)]. These enzymes serve as links to the existing (constitutive) triose isomerase (EC 5.3.1.1.) and aldolase (EC 4.1.2.13), and thus a glucogenic segment leading from glycerol to FDP is created, and, as with other examples given before, FDP inhibits by feedback the first enzyme (glycerol kinase) of this pathway (145). Similarly, when enteric bacteria are growing on a C_4 acid as the sole carbon source, pyruvate is produced by decarboxylation of malate by an inducible triphosphopyridine nucleotide (TPN)-specific malic enzyme (EC 1.1.1.40), and this enzyme, in effect, becomes the first enzyme of the pathway leading from malate to acetyl-CoA. In accord with the principles of end-product control, acetyl-CoA inhibits the TPN-specific malic enzyme by an allosteric competition with the substrate, malate (108).

There is suggestive evidence that end-product control also operates in the segment between FDP and glucose-6-phosphate during glucogenesis. Bacteria, like higher organisms (119), use the first enzyme of this sequence, fructose diphosphatase (EC 3.1.3.11) to produce fructose-6-phosphate which is then converted into glucose-6-phosphate by hexose isomerase (EC 5.3.1.9). Fructose diphosphatase is produced constitutively in *E. coli* and is of no significance whatsoever when glucose or its precursors are the growth substrates (27), as is clearly demonstrated by the fact that *E. coli* mutants lacking this enzyme grow normally on glucose. Indeed, the functioning of the enzyme during active glycolysis would be deleterious to

the organisms because, if left uncontrolled, it would convert FDP produced by phosphofructokinase (Fig. 1) back to fructose-6-phosphate. Part of this problem of metabolic short-circuiting is solved in *E. coli*, and in higher organisms, by having an energy-linked signal (adenine nucleotides) produce opposite effects on the two substrate-coupled enzymes, phosphofructokinase and fructose diphosphatase. The former enzyme in *E. coli* is activated (6, 8), and the latter is inhibited by adenosine diphosphate (ADP) or adenosine monophosphate [AMP (28)]. However, when the cell is energy saturated (i.e., when the ATP/AMP ratio is high), this control system is of minimal significance because the ADP and AMP levels are expected to be low under such conditions (*see below*). Fraenkel (26) recently demonstrated that in *E. coli* glucose-6-phosphate inhibits fructose diphosphatase and this negative feedback possibly restricts the conversion of FDP to fructose-6-phosphate during glycolysis. This feedback system is not expected to be effective during growth on a glucogenic substrate (such as glycerol) because the steady-state levels of the inhibitor (glucose-6-phosphate) are considerably reduced under such conditions (46, 92). This control has been demonstrated to operate in vivo. Using a mutant of *E. coli* which lacks both hexose isomerase and glucose-6-phosphate dehydrogenase (EC 1.1.1.49), it was demonstrated (26) that growth of this mutant on glycerol is inhibited drastically in the presence of small amounts of glucose, i.e., under conditions when the concentration of glucose-6-phosphate increases.

Precursor Activation

From the physiological point of view, a control mechanism unique so far to amphibolic pathways is precursor activation (43, 106, 109). Operationally, this control is symmetrically opposite of end-product control. In the case of the latter the last metabolite of a pathway inhibits the first enzyme, whereas in precursor control the first metabolite of the sequence activates the last enzyme of that sequence. In all known cases, the activated enzyme catalyzes a reaction which is practically irreversible under physiological conditions. Precursor activation was first discovered by Leloir and co-workers (64, 65) who demonstrated that mammalian glycogen synthetase (EC 2.4.1.11) was powerfully activated by glucose-6-phosphate, an amphibolic precursor of glycogen. Since then a number of such positive "feedforward" loops have been found in bacteria and some fungi, but only in amphibolic pathways. In the enteric bacteria, the intermediate which occupies a central position in this regard is FDP. As in the case of end-product control, one can delineate arbitrarily a number of metabolic segments where precursor activation operates.

(i) FDP → → → PEP → oxalacetate
(ii) FDP → → → PEP → pyruvate
(iii) PEP → pyruvate → → → acetyl-CoA
(iv) FDP → fructose-6-phosphate → glucose-1-phosphate → adenosine diphosphate glucose (ADP-glucose)

In each of these cases, the enzyme activated by the first-named metabolite is the one preceeding the last-named intermediate. It is interesting to note that FDP activates pyruvate kinase (EC 2.7.1.40) (sequence ii) and PEP carboxylase (sequence i), both of which are located at a branch point (Fig. 1) and use PEP as a substrate. This circumstance, as will be seen later, creates a number of complications in the regulation of glycolysis for which the enteric organisms have evolved ingenious and novel types of control mechanisms. Because FDP activates pyruvate kinase from a variety of sources, including yeast (43) and mammals (7, 121, 123), it would seem logical to assume that this control may have arisen relatively early during evolution. Perhaps the same is true of the activation of ADP-glucose pyrophosphorylase by FDP (sequence iv). Preiss and co-workers (90, 91) demonstrated this control not only in a number of bacteria (*Aerobacter aerogenes*, *Arthrobacter*, *Micrococcus lysodeikticus*, and *E. coli*) but also in green plants. As was mentioned before, glycogen synthesis in mammalian cells is controlled by modulation of glycogen synthetase, whereas in bacteria and plants it is controlled by modulation of ADP-glucose pyrophosphorylase which converts glucose-1-phosphate into ADP-glucose (36). The latter is the glucosyl donor for α-1,4-glucan synthesis. The ADP-glucose pyrophosphorylase of *E. coli* is activated not only by FDP but also by glyceraldehyde-3-phosphate and PEP, although FDP is the activator par excellence. It appears that easily interconvertible precursor pools rather than a specific precursor are the control signals here. An entirely analogous situation is known to occur in some biosynthetic pathways. The regulatory signal for aspartate transcarbamylase (EC 2.1.3.2), the first enzyme of the pyrimidine pathway in *E. coli*, for instance is provided by the nucleoside triphosphate pools, although cytidine-5'-triphosphate (CTP) is the most potent inhibitor (32, 33).

The precursor activation loops discussed above, although typical of the enteric forms, are not the only ones found in bacteria. As an illustration of this point, lactate dehydrogenase (EC 1.1.1.27) from several members of the genus *Streptococcus* is strongly and specifically activated (139) by FDP, but this precursor activation does not occur

in *E. coli* or *Butyribacterium rettgeri* (122, 138). This example, among others to be discussed later, illustrates that the "unity of biochemistry" concept which has been so useful in the study of other biochemical phenomena has paid little dividends in the field of controls. Unity of principles underlying controls certainly exists, but the manifestations are necessarily as divergent as the diversity of the organisms.

Energy-Linked Controls

As stated earlier, energy-linked controls (to the exclusion of end-product or precursor control) are characteristic of strictly catabolic or degradative pathways. It is, therefore, not surprising that amphibolic routes which fulfill both a catabolic and anabolic function are also controlled by compounds which are energy donors or energy acceptors of a cell. Generally speaking, the compounds of greatest interest in this regard are AMP, ADP, pyrophosphate, adenosine triphosphate (ATP), and inorganic phosphate. However, since various other pyrimidine or purine nucleoside mono-, di-, and triphosphates are readily interchangeable with adenine nucleotides by means of the ubiquitous nucleoside kinases, most of which have an equilibrium constant near unity, there is a possibility that nucleotides other than the adenylates may serve as indicators of the energy state of the cell.

The amphibolic channels provide not only hydrogen for the electron transport chain, which generates energy in the form of ATP, but also the carbon skeletons needed for biosynthesis. The ATP produced is used up by the biosynthetic pathways and other energy-utilizing processes of the cell with the generation of ADP or AMP. Thus, when the supply of energy is low adjustments must occur to speed up the ATP-generating reactions and, conversely, when the energy supply is high these reactions must be slowed down. Bacteria, like other organisms (3, 61), achieve this by modulating the activities of crucial, rate-limiting steps of the sequences which are involved in ATP generation (*see* Fig. 1). Thus, in *E. coli*, phosphofructokinase is activated by ADP and GDP (8), one of the two pyruvate kinases (*see* later) is activated by AMP (72), pyruvate dehydrogenase component of pyruvate dehydrogenase complex is activated by GDP and AMP (110), and fructose diphosphatase is inhibited by AMP and ADP (28). The significance of activation by AMP (or ADP) of the first three enzymes mentioned above lies in the fact that for the regulation of glycolytic and terminal oxidation sequence, AMP (and to a lesser extent, ADP), as first suggested by Krebs (61), is a sensitive indicator of the state of ATP supply because of the intervention of adenylate kinase (EC 2.7.4.3). This enzyme is present in large concentrations in most microbial cells and has an equilibrium constant [(AMP) (ATP)/(ADP)^2] of 0.44 (23). If the total concentration of adenylates in a bacterial cell, such as that of *E. coli*, is assumed to be 4 mM, the concentration of ATP, ADP, and AMP equilibrium will be 3, 0.9, and 0.1 mM, respectively. It can be readily calculated that when the concentration of ATP drops to 2 mM (33%), the concentration of ADP rises to 1.5 mM (67%) and that of AMP rises to 0.5 mM (500%). It is reasonable to assume, then, that the level of AMP, more than the absolute concentration of ATP or ATP/ADP, is a sensitive control signal (61). Any decrease in the supply of ATP will be reflected in the stimulation of enzymes which are involved either directly in the generation of ATP (pyruvate kinase) or which furnish products which may be used for energy generation through the terminal oxidative pathway. Atkinson (5) recently suggested that a balance between the concentrations of the adenylates may be an important regulatory parameter for all enzymes which consume or produce ATP whether or not they have regulatory sites accommodating specific adenine nucleotides.

Control of Duplicate Enzymes

So far, the discussion has centered around the generalities of signals utilized for the control of enzymes of the amphibolic pathways. At this point, it is necessary to digress a little and examine the strategy employed by the cells to manage the problems of metabolic traffic which arise when the cells switch over from growth conditions under which glycolysis predominates (growth on glucose) to those under which glucogenesis is essential (for instance, growth on C_3 or C_4 compounds). Glycolysis shares with glucogenesis the block of freely reversible, constitutive enzymes producing FDP from PEP. For glucogenesis to occur, the bacterial cells induce enzyme systems which lead to the formation of PEP. In the enteric bacteria, the two enzymes which are capable of doing so are PEP carboxykinase (EC 4.1.1.32) and PEP synthase; the former is induced (45, 143) when a C_4 compound (succinate, malate) serves as a carbon source and the latter only when a C_3 compound (alanine, pyruvate, or lactate) is the energy source (17). In both cases, PEP is produced at the expense of ATP. Since the cells contain pyruvate kinase, PEP produced during glucogenesis could easily be converted to pyruvate and ATP instead of giving rise to triose phosphate. This would be wasteful not only because the synthesis of hexoses would be curtailed but also because there would be an unnecessary accumulation of pyruvate which during glucogenesis is produced

by the TPN-specific malic enzyme. This enzyme is known to be derepressed during growth of *E. coli* on succinate or malate (48; 103). To prevent this kind of short-circuiting, the enteric bacteria, like mammalian tissues (120), produce two pyruvate kinases, one activated by FDP and derepressed during growth on glucose (74) and the other activated by AMP and produced constitutively (Waygood and Sanwal, *unpublished data*). The FDP-activated pyruvate kinase probably does not function during growth on succinate (i.e., during glucogenesis) because of repression and also because the concentration of the activator (FDP), like other hexose phosphates, falls to a level almost 25% of that found during vigorous growth on glucose (46; Sanwal, *unpublished data*). Similarly, the AMP-activated pyruvate kinase functions during glucogenesis under restrictive conditions because AMP activation in molecular terms may be considered equivalent to ATP inhibition, and the functioning of this pyruvate kinase is thus dependent on the energy balance of the cell.

A second instance in which duplication of enzymes occurs in the enteric bacteria is in the conversion of malate to pyruvate. The two enzymes involved are the TPN- and the diphosphopyridine (DPN)-specific (EC 1.1.1.38) malic enzymes (48). The equilibrium constants of both of the enzymes are such that they favor formation of pyruvate, and this is also their probable function in vivo. As with the two pyruvate kinases discussed above, again the presence of two malic enzymes may be viewed as a device which directs an orderly metabolic flow depending upon the carbon source being utilized by the cells. Thus, when glycolysis is predominant, the primary source of pyruvate (and ATP, *see below*) is PEP, and it is desirable to prevent an uncontrolled flow of the same compound (pyruvate) into the central pool through malate. This is accomplished in *E. coli* by the repression of the TPN-specific malic enzyme (103) during growth on glucose (catabolite repression) and the inhibition of its activity by reduced diphosphopyridine nucleotide (DPNH) (104), a compound which accumulates during glycolysis (Table 1) and, as we will see presently, is a central control signal for a larger number of metabolic sequences. For the same possible reasons, the activity of the second, constitutive, malic enzyme (DPN-dependent) is inhibited by CoA and this inhibition is relieved specifically by aspartate (98). In physiological terms, this means that the DPN-specific malic enzyme is formed by the enteric bacteria for the degradation of C_4 acids and it functions only when there is an accumulation of these compounds. The amount of C_4 acids degraded through this enzyme, however, is further regulated by the state of the energy supply of the cell, which is reflected in the inhibition of the enzyme by ATP in a manner that cannot be relieved by the activator aspartate (98). It is not clear at this stage, however, why the presence of two enzymes is required to achieve the same end, unless it be that one of the enzymes (TPN dependent) has a function in addition to the formation of pyruvate, namely, the generation of reduced triphosphorpyridine nucleotide (TPNH) for biosynthetic purposes.

TABLE 1. *Concentration of DPN and DPNH under different conditions of growth*[a]

Growth phase	Carbon source	DPN	DPNH	DPN/DPNH
Early log	Glucose	2.5	5.5	0.45
	Succinate	3.2	3.2	1.00
Mid log	Glucose	2.5	3.0	0.82
	Succinate	2.9	2.0	1.45
Stationary	Glucose	2.1	4.5	0.48
	Succinate	3.2	3.1	1.00

[a] *E. coli* B cells were preadapted to glucose or succinate by growing them for at least 10 generations in the appropriate medium. Fernbach flasks containing 1 liter of mineral salts medium (with glucose or succinate as carbon source) were inoculated with a suspension of pregrown cells and shaken vigorously at 37 C. Growth was monitored by measuring cell density in a Klett photometer. At appropriate times, 500 ml of the culture was centrifuged and analyzed for coenzyme content by enzymatic procedures. Values in the table are reported as micromoles per gram, dry weight.

Recent evidence indicates that duplicity of enzymes also occurs in the initial segment of the tricarboxylic acid cycle. Two TPN-specific isocitrate dehydrogenases (EC 1.1.1.42) have been reported to occur in *Salmonella* and *E. coli*, one of which is inhibited by ATP, ADP, or guanosine triphosphate (GTP) (77). Two TPN-dependent glutamate dehydrogenases (EC 1.4.1.3.) have also been separated from *E. coli* (Lo and Sanwal, *unpublished data*). As was pointed out by Umbarger and Brown (128, 129), and as has been the experience so far, whenever two enzymes are found to catalyze the same reaction in a cell their metabolic roles are quite different, and it is likely that the duplicate enzymes referred to above will be found to be regulated differently from each other.

Interaction of Feedback Loops in Diverging Channels

It was mentioned at the outset that the intermediates of amphibolic pathways serve a number of functions and some of these are not entirely related. This will be clear from a study of Fig. 2. An intermediary metabolite such as PEP for

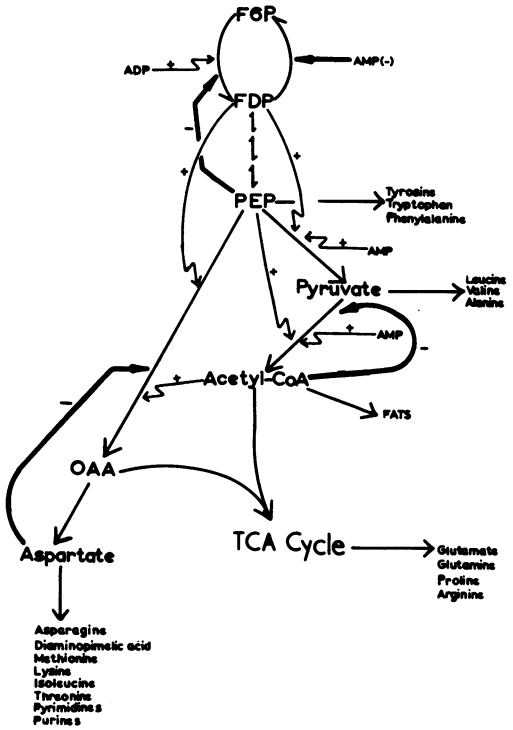

FIG. 2. *Cascade type of regulation of glycolysis in E. coli. Plus and minus signs indicate activation or inhibition, respectively, by the various metabolites.*

instance, not only is used for the biosynthesis of the aromatic amino acids (tryptophan, tyrosine, phenylalanine) but also must be converted into pyruvate, on the one hand, through the catabolic channel (catalyzed by pyruvate kinase), and to oxalacetate, on the other, through the anaplerotic channel (catalyzed by PEP carboxylase). In other words, PEP must be used to satisfy both the energy and biosynthetic requirements of the cells. Now, biosynthesis can only proceed at a constant rate if there is sufficient supply of energy (in the form of ATP) and reducing power. Since the supply of ATP must decrease as energy-consuming, biosynthetic processes proceed and since the energy supply can only be replenished by the oxidation of C_3 compounds, it follows that the amounts of PEP withdrawn via the catabolic channel must fluctuate considerably. This demands that mechanisms should be available which control the distribution of PEP into the anaplerotic and the catabolic channels. In the enteric bacteria, the enzymes which produce oxalacetate and pyruvate are both controlled in an allosteric manner by the substrate (PEP), which typically yields sigmoid saturation curves (72, 73). The K_m of PEP is not the same, however, for the two enzymes. For PEP carboxylase, it is $\backsim$ 10 mM (101) and for the FDP-activated pyruvate kinase it is $\backsim$ 3.5 mM (Waygood and Sanwal, *unpublished data*). This means that PEP carboxylase must be virtually inactive in the cell. Following the work of Keech and Utter (52, 53) with ATP-dependent pyruvate carboxylase (EC 6.4.1.1.) (of animals), it has now been demonstrated that in the enteric bacteria (10, 70), acetyl-CoA activates PEP-carboxylase powerfully; at saturation it brings the K_m for PEP down to 1 mM. Activation of PEP carboxylase seems to be a mechanism which ensures that a supply of oxalacetate will be forthcoming to enable the oxidation of acetyl-CoA (by condensation with oxalacetate to citrate) through the citric acid cycle. However, oxalacetate is required not only for condensation with acetyl-CoA but, provided that energy is available, also for the biosynthesis of proteins, pyrimidines, and purines after its conversion to aspartate by aspartate-glutamate transaminase (EC 2.6.1.1). Now, in a bacterial cell, the levels of acetyl-CoA must be highly variable because not only is this compound diverted to the synthesis of fats but its own synthesis by the pyruvate dehydrogenase complex is modulated (*see* Fig. 2) by the state of energy supply. (AMP activates pyruvate dehydrogenase component and in molecular terms this is equivalent to inhibition by ATP.) If activation only by acetyl-CoA were an indispensable condition for the production of oxalacetate, a constancy of its supply commensurate to the demands of protein, pyrimidine, and purine biosynthesis could not be maintained. However, activation by FDP of PEP carboxylase, discussed earlier, perhaps alleviates this difficulty. It can be shown that acetyl-CoA and FDP together bring about a *cooperative* activation (73) of PEP carboxylase; i.e., the percentage activation in the presence of acetyl-CoA and FDP together is much higher than the sum of percentages of activation in the presence of each activator alone (Fig. 3). This means, in physiological terms, that FDP compensates for any decrease in the degree of activation of PEP carboxylase brought about by a decrease in the supply of acetyl-CoA, and vice versa. In other words, the cooperative activation is a necessary stabilizing device as far as the formation of oxalacetate is concerned, and helps to ensure that the biosynthetic function of glycolysis is not seriously impeded when momentary adjustments are occurring in the competing catabolic channels in response to changes in energy levels. To clarify this a little further, consider a situation where momentarily the ATP supply is high and acetyl-CoA is being converted into fatty acids. Under these conditions, the right-hand channels of Fig. 2 leading from FDP via PEP to acetyl-CoA are probably not functioning optimally be-

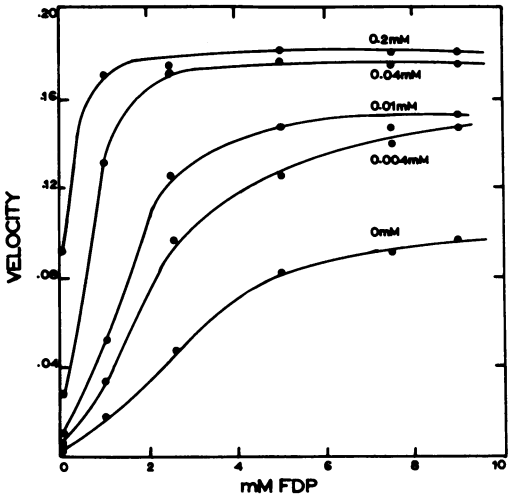

FIG. 3. *Interactions of fructose-1, 6-di-P and acetyl-CoA in the activation of PEP carboxylase from Salmonella typhimurium. The numbers above the lines represent the concentration of acetyl-CoA* (73).

cause they require ADP (phosphofructokinase) or AMP (pyruvate dehydrogenase system and AMP-activated pyruvate kinase) for maximal activity. As was pointed out earlier, when ATP concentration is high the concentration of ADP and AMP is low. As the concentration of ATP decreases in proportion to acetyl-CoA consumed in fatty acid synthesis, the concentration of AMP and ADP rises and triggers a cascade-type of activation of the catabolic channels due to the combined action of strategic, interdependent, energy-linked, and precursor activation loops (Fig. 2). Thus, ADP activates phosphofructokinase, which results in enhanced levels of FDP. It is expected that this augmented concentration of FDP will compensate for the momentary decreases in the concentration of acetyl-CoA in the stimulation of PEP carboxylase. At the same time, the excess FDP activates one of the two pyruvate kinases (FDP-activated) and AMP activates the other one. The PEP formed from FDP activates the pyruvate dehydrogenase complex along with AMP with the net result that both ATP (from pyruvate kinase) and acetyl-CoA levels are replenished. Again, the decrease in the FDP concentration is compensated for by augmented levels of acetyl-CoA in the activation of PEP carboxylase. It is recognized in this discussion that what has been stated above is, in our present state of knowledge, only a reasonable guess and has necessitated some ad hoc assumptions. For instance, the concentration of ATP in vivo may momentarily decrease not only because of the utilization of acetyl-CoA but also because of the operation of other ATP consuming biosynthetic processes, and one can easily visualize a situation in which the concentrations of FDP and acetyl-CoA decrease simultaneously. Further safeguards seem to have been provided for such eventualities to ensure functioning of the biosynthetic arm. Thus, PEP carboxylase is activated by CMP, cytidine diphosphate (CDP), and GTP (but not by ATP, ADP, or AMP), and this activation, like FDP, is again cooperative with acetyl-CoA (101). It is not entirely clear at the present time what relationship the cytidine nucleotides may have to the metabolic fluctuations in the levels of acetyl-CoA which necessitate the occurrence of the aforementioned cooperative activation. This fact, however, does not deter from the importance of the argument that when a metabolite is used for both biosynthetic and catabolic purposes, and when, of necessity, the amount of that metabolite passing through the catabolic channel fluctuates in response to energy adjustments, controls, however complex, have to be made available which ensure that a constant amount of the shared metabolite goes into the biosynthetic channel. This argument has heuristic value. Bacteria do differ with regard to the location of branch points in amphibolic pathways. In *Pseudomonas* and *Arthrobacter*, PEP carboxylase seems to be absent and oxalacetate is produced, as in yeast (68) and animals (52), by the ATP-dependent pyruvate carboxylase. It is at these points that novel types of controls are likely to be found.

It was stated earlier that all of the activating controls of PEP carboxylase of enteric bacteria have their origin in the fact that this enzyme would otherwise be virtually nonfunctional in vivo because of its low affinity for PEP. One might ask why evolution has selected for an enzyme of this nature. A reasonable answer is perhaps to be found in the relative ease with which the presence of such an enzyme allows bacteria to switch from one carbon source to another. PEP carboxylase is only important when growth occurs on glucose, glycerol, or pyruvate, but it is a hindrance when a C_4 compound is being utilized. Because of the already high K_m for PEP, it is relatively easy to inhibit the enzyme almost completely by a negative feedback of its activity by C_4-dicarboxylic acids (73; Fig. 2).

REDUCED COENZYMES AS CONTROL SIGNALS

Regulation of Converging Channels

It must be amply clear from the above account that quite an unorthodox strategy is employed by the cells to control metabolic flow in diverging channels. Simple metabolic control mechanisms

such as end-product inhibition, precursor activation, etc. are not sufficiently sophisticated to fulfill regulatory demands at branch points of amphibolic pathways. As will be seen presently, the same seems to hold true for converging sequences. In the enteric bacteria, a converging sequence consists of enzyme systems which lead from oxalacetate to pyruvate via malate dehydrogenase (EC 1.1.1.37) and malic enzyme (Fig. 1, 4). The problem of control in this area can best be understood, if the question is asked: when oxalacetate is produced by PEP carboxylase in response to a particular need, namely, to enable oxidation of acetyl-CoA or to supply the biosynthetic routes with aspartate, what mechanism determines that it will only be utilized for the purpose it was produced? In the absence of such a mechanism, oxalacetate could conceivably be easily diverted to malate and thence to pyruvate (through the converging channel; Fig. 4), and the latter would then be available for the generation of more acetyl-CoA. This would be wasteful in view of the fact that a controlled quantity of pyruvate is already produced from PEP by pyruvate kinase. The problem as outlined here is rather unique to bacteria because, here, compartmentation appears to be in a relatively rudimentary form. In eucaryotes, the same difficulty in regulation exists but it seems to have been overcome by imposing rigid compartmentation of certain enzymes and metabolites inside mitochondria. Consider the formation of oxalacetate in mitochondria when acetyl-CoA levels are high. As has been mentioned before, activation of the ATP-dependent pyruvate carboxylase by acetyl-CoA results in the formation of enough oxalacetate to permit the oxidation of acetyl-CoA (52). The resulting excess oxalacetate is probably not utilized by mitochondrial malate dehydrogenase owing to its inhibition by the substrate, nor is it immediately accessible to the extramitochondrial malate dehydrogenase owing to the problems associated with permeation, etc. (14, 113). This sort of compartmentation reasonably assures that the surplus of oxalacetate will mostly be utilized for the purpose it was produced, namely, to condense with acetyl-CoA. Admittedly, this is an extremely simplified version of the actual, and definitely more elaborate, controls available in the partitioning of metabolites (113) between mitochondria and cytosol, but it serves to illustrate the fact that in the absence of compartmentation the problems of enzymatic regulation in this area are unique to bacteria.

Recently it has been found that the main control signal in converging channels in *E. coli* and *S. typhimurium* is DPNH. This coenzyme specifically inhibits, in an allosteric manner, the activity

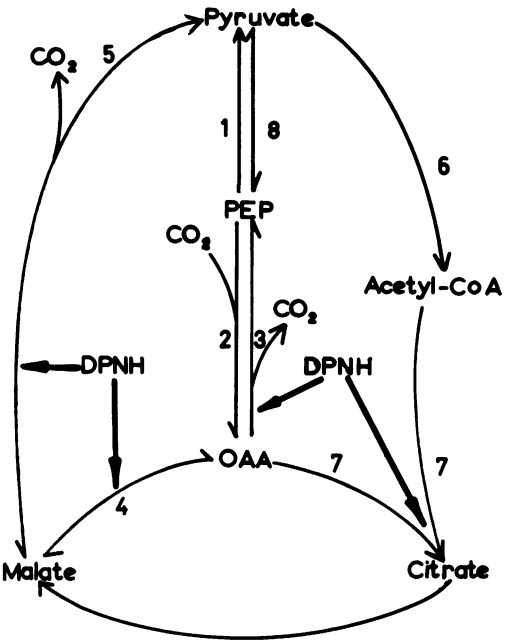

FIG. 4. *Enzymic reactions inhibited by DPNH (shown by heavy arrow):1 = pyruvate kinase; 2 = PEP carboxylase; 3 = PEP carboxykinase; 4 = malate dehydrogenase; 5 = TPN-specific malic enzyme; 6 = pyruvate dehydrogenase complex; 7 = citrate synthetase; 8 = PEP synthase.*

of malate dehydrogenase and malic enzyme along with that of a large number of other enzymes (*see below*). If oxalacetate produced in response to activation of PEP carboxylase by acetyl-CoA and FDP is to be prevented from passing into the converging channel (Fig. 2, 4), malate dehydrogenase and malic enzyme must be blocked. The malate dehydrogenase of *E. coli* seems to be designed in such a way that, in the presence of increasing concentrations of DPNH, a continually steep sigmoidal velocity response is given by oxalacetate as a substrate with a concomitant decrease in the V_{max} of the reaction (97). This means, in physiological terms, that provided the DPNH concentration is high, oxalacetate has a threshold concentration below which it cannot be easily converted to malate. Even more stringent is the control of malic enzyme (103) which is allosterically inhibited by oxalacetate (K_i apparent = 0.01 mM), DPNH, TPNH, acetyl-CoA and cyclic 3′-5′-AMP (105). The importance of the inhibition of this enzyme by oxalacetate is obvious in view of what has been discussed before about the lack of rigid compartmentation and its possible consequences. Thus, when oxalacetate concentration is high in response to a specific metabolic demand, inhibition of competing enzymes prob-

ably ensures the use of oxalacetate to fulfill that demand.

Regulation of the Citric Acid Cycle by DPNH

It was suggested by Atkinson (3) that the entry of acetyl-CoA into the citric acid cycle may be regulated by ATP. The suggestion was based on the finding that mammalian (47) and yeast citrate synthetases (87) are inhibited by this compound. This has been found to be true also for some strictly aerobic bacteria like *Bacillus subtilis* (25). In the enteric forms, however, the citric acid cycle seems to be controlled by DPNH. Weitzman (131) first showed that citrate synthetase of *E. coli* is powerfully inhibited by the reduced coenzyme in a specific manner. With the finding that malate dehydrogenase is also inhibited by DPNH (97), there is very little doubt that DPNH indeed may be the central control signal for the regulation of citric acid cycle. It may then be asked why DPNH is, to the exclusion of other possible compounds (such as adenine nucleotides which are the energy indicators of a cell), an allosteric inhibitor of the two crucial enzymes of the energy-generating pathway. It has been suggested (131, 132) that this inhibition may be a kind of feedback control by end product, since DPNH is generally the product of the dehydrogenating reactions of the citric acid cycle. This explanation is probably not the right one for the enteric bacteria because, first, so far as is known, DPN is reduced only at one step of the cycle, namely, the step catalyzed by α-ketoglutarate dehydrogenase (EC 1.2.4.2) and, second, as will be discussed later, DPNH inhibits in an allosteric manner the activity of several other enzymes which have no connection whatsoever with the citric acid cycle (Fig. 4).

The reason for the inhibition of citric acid cycle by DPNH has rather to be sought in a biochemical oddity of the enteric bacteria and probably other facultative anaerobes as well. Despite the extensive use of *E. coli* in the study of intermediary metabolism and biochemical phenomena in general, this organism is atypical so far as its terminal respiration is concerned. The enteric bacteria live largely by anaerobic glycolysis even when growing on glucose aerobically. The citric acid cycle predominantly serves a biosynthetic function under these circumstances and is perhaps interrupted at the level of succinic dehydrogenase (EC 1.3.9.1). These statements are based on the observation that a number of crucial enzymes such as succinic dehydrogenase, succinic thiokinase (EC 6.2.1.5), α-ketoglutarate dehydrogenase, and cytochromes are repressed in the presence of glucose (2, 93). Mutants which lack succinic dehydrogenase (44) ubiquinones and vitamin K [required for oxidative phosphorylation (19)] grow well aerobically on glucose. It is for these reasons, also, that unlike eucaryotes and possibly other strictly aerobic bacteria, the enteric organisms have a preponderance of DPNH when aerobic growth occurs on glucose. It can be demonstrated that the DPN/DPNH ratio is on the average 0.5 in glucose-grown cells as compared with 1.0 in the case of succinate-grown cells (Table 1). The level of DPNH can thus be considered as an indicator of the state of glycolysis. Since ATP must obviously be generated, albeit in an uneconomical manner, primarily by glycolysis during growth on glucose, it is easy to see the purpose of the inhibition of citrate synthetase by DPNH. This inhibition would minimize the production of more DPNH, through α-ketoglutarate dehydrogenase, for example, under conditions in which a supply of this compound is already available in excess of that which can be handled by the repressed electron transport chain. However, it may be noted that functioning of citrate synthetase is important, if for nothing else to provide glutamate by further transformations of citrate, which in turn must be available to aminate oxalacetate. Accordingly, it has been found that acetyl-CoA and oxalacetate deinhibit the enzyme against DPNH and α-ketoglutarate, respectively (142).

Before going any further, a discussion as to why *E. coli* and related facultative anaerobes have evolved an apparently wasteful way of utilizing glucose aerobically, namely, by glycolysis alone, would be useful. Amarsingham and Davis (2) advanced the ingenious argument that this mode of growth gives selective advantage to the enteric bacteria in competition with other microbes. Thus, incompletely oxidized intermediates to which the competing organisms may not adapt readily are quickly produced from glucose, and these may remain in the external milieu for use as soon as the supply of glucose is exhausted.

Most of the arguments concerning regulation so far given have, of necessity, been based on observations made with the enteric bacteria, if only for the reasons that these organisms have been popular with biochemists and physiologists alike and that a level of information is available which allows some reasonable guesses to be made regarding integration of control circuits. Nevertheless, the question may be asked whether control by DPNH is a general property of bacterial systems. This question also relates to the contention made earlier that part of the complex controls utilizing DPNH as inhibitor are necessitated in bacteria by the absence of rigid compartmentational controls. In a recent study, Weitzman and Jones (134), using inhibition of citrate synthetase by DPNH as a criterion, demonstrated that all of

the 18 genera of gram-negative bacteria (including *Azotobacter*, *Pseudomonas*, *Moraxella*, *Flavobacterium*, etc.) tested had a citrate synthetase susceptible to inhibition, whereas the enzyme from aerobic gram-positive bacteria (among which are *Micrococcus*, *Staphylococcus*, *Bacillus*, and *Arthrobacter*) was not affected by DPNH. Within the DPNH-susceptible class, subclasses could be differentiated on the basis of the capability of AMP to reverse DPNH inhibition. It has been suggested (134) that the enzyme systems of bacteria showing relatively simple mesosomal organization may be more susceptible to DPNH inhibition, whereas those associated with complex mesosomal organization are not affected by DPNH. There is little information available, however, about details of mesosomal structure in bacteria, such as is needed to document such a proposal.

Control of Glucogenesis

Glucogenesis from C_3 and C_4 compounds in both eucaryotes and procaryotes is made possible by the presence of enzyme systems other than pyruvate kinase which convert a number of compounds to PEP (Fig. 4). It is now well established that the ubiquitous pyruvate kinase is incapable of producing PEP from pyruvate owing to its near irreversibility. In *E. coli* the two main enzymes which make glucogenesis possible from C_4 substrates are PEP carboxykinase and TPN-specific malic enzyme, both of which are induced to a considerable extent during growth on C_4 compounds such as succinate and malate (103, 143). Loss of PEP carboxykinase by mutation prevents growth on succinate, malate, fumarate, and acetate, which proves the indispensibility of this enzyme for glucogenesis on these substrates (45). Such mutants, however, grow on pyruvate or lactate and it has been now demonstrated by Cooper and Kornberg (17) that this is made possible by the induction of PEP synthase which is the main glucogenic route during growth on C_3 compounds.

It was shown recently that both PEP carboxykinase (143) and TPN-specific malic enzyme (103, 104) are inhibited in an allosteric manner by DPNH. Whereas the latter enzyme is also susceptible to inhibition by TPNH, acetyl-CoA, and cyclic 3′,5′-AMP (103, 105), the inhibition of the former enzyme by DPNH is strictly specific (143). The specificity of the control for the two enzymes, of course, reflects the specificity of their functions. Whereas the function of PEP carboxykinase is restricted exclusively to the formation of PEP by an ATP-dependent decarboxylation of oxalacetate, the functions of malic enzyme are diverse, as for instance, formation of TPNH for reductive synthesis and formation of acetyl-CoA via pyruvate during glucogenesis. The allosteric control of the glucogenic enzymes by DPNH can be considered as a mechanism whereby unnecessary glucogenesis is prevented when vigorous growth is occurring on glucose. It has been pointed out before that in the enteric bacteria levels of DPNH are indicators of the state of glycolysis, and it stands to reason that they be also the signals which hinder the functioning of enzymatic routes which are opposite to glycolysis.

Regulation of Oxidative Pentose Pathway

One of the enigmatic problems perhaps central to the understanding of carbohydrate metabolism in bacteria is the nature of the control system (or systems) which determines the distribution of glucose-6-phosphate into the pentose phosphate pathway, on the one hand, and the glycolytic pathway on the other. It is well known that pentose is synthesized in bacteria by both the oxidative and the nonoxidative pathways. In the former, 1 mole of glucose-6-phosphate is converted to 1 mole of pentose, 1 mole of carbon dioxide, and 2 moles of TPNH, whereas in the latter 5 moles of hexose phosphate is converted nonoxidatively into 6 moles of pentose phosphate. Numerous studies with ^{14}C-labeled glucose (50, 51) have demonstrated that 20 to 30% of glucose is utilized by the enteric bacteria through the oxidative pentose pathway. There is a distinct possibility, however, that the major function of this pathway is not to generate pentose for biosynthetic purposes. This statement is based on the finding, among others, of Caprioli and Rittenberg (11) that nucleosides isolated from cells of *E. coli* grown on *l*-^{18}O-glucose have all of the ^{18}O label in the 5′-position. Similarly, Katz and Rognstad (49) showed that only a very small amount of glucose carbon passing through the oxidative pathway in *E. coli* is converted into phosphoribosyl pyrophosphate. In view of these observations, there is a distinct possibility that one of the primary functions of the oxidative pentose pathway is to generate TPNH for reductive biosynthesis. Model and Rittenberg (79), on the basis of their finding that the amount of glucose metabolized through the oxidative pathway drops to about 50% during stationary compared to the logarithmic phase, have suggested that the oxidative pathway is regulated by the availability of oxidized TPN, and this is also the opinion of other workers (22). It has now been demonstrated (99) that the activity of glucose-6-phosphate dehydrogenase of *E. coli* is inhibited in an allosteric manner by DPNH. It is indeed extremely interesting that in *E. coli*, DPNH controls all of the TPNH-generating pathways of the cell. The two

main enzymes that produce TPNH, namely, TPN-specific malic enzyme and glucose-6-phosphate dehydrogenase, are directly inhibited by DPNH, and the activity of the third enzyme, TPN-specific isocitrate dehydrogenase (*E. coli* not possessing a DPN-specific enzyme), is indirectly controlled by an inhibition of citrate synthetase which is the only enzyme supplying isocitrate in the enteric bacteria. It is germane to point out, however, that the control of glucose-6-phosphate dehydrogenase by DPNH is not of universal occurrence in bacteria. In *P. aeruginosa*, for instance, this enzyme shows completely different characteristics and is inhibited by ATP, much like the enzyme from mammalian tissues.

Control of Glycogenesis

Glycogenesis, as mentioned elsewhere, is controlled in bacteria at the level of ADP-glucose synthesis (36). In animals, it is controlled both at the level of glycogen synthesis and breakdown (glycogenolysis) by the regulation of glycogen synthetase (65) and phosphorylase (EC 2.4.1.1), respectively. The glycogen phosphorylase in bacteria, to the contrary, seems not to be regulated by allosteric mechanisms at all (16). The importance, again, of reduced pyridine nucleotide coenzymes in the control of glycogenesis in *E. coli* is demonstrated by the recent finding of Preiss and coworkers (91) that ADP-glucose pyrophosphorylase is powerfully activated by TPNH. This control is exerted on the enzyme in addition to that caused by triose phosphates discussed earlier. Preiss and co-workers (91) view the activation by TPNH as a device by which the flux of carbon is diverted towards glycogen synthesis during limited growth, such as is expected to occur in the stationary phase during which the concentration of TPNH is expected to increase because it is not consumed in biosynthetic reactions.

Control of Other Enzymes by DPNH

It is clear from the account given above that the reduced pyridine nucleotide coenzymes play a large part in the regulation of amphibolic pathways. Indeed, the enzymes discussed before are not the only ones controlled by reduced coenzymes. Phosphotransacetylase (EC 2.3.1.8) from *E. coli*, for instance, is powerfully inhibited by DPNH (116) and, judging from the kinetic evidence presented by Suzuki et al. (116), this seems to be an allosteric type of inhibition. Similarly, it has been shown that (37) the dihydrolipoyl dehydrogenase component of the pyruvate dehydrogenase complex of *E. coli* is inhibited by DPNH. In this case, however, it is not clear whether this is product inhibition (a property of all enzymes because the substrate and the product are expected to bind at the same site on the enzyme surface and cause inhibition by steric hinderance) or allosteric inhibition.

Much of the information about reduced pyridine coenzyme-linked controls has been accumulated primarily from a study of enzymes of *E. coli* and other enteric bacteria. It does, however, seem that unconventional controls of this nature will be found to be of more common occurrence than hitherto realized. As an example, it has recently been demonstrated that the reductive pentose cycle in *Hydrogenomonas* (69) and in *Rhodopseudomonas spheroides* and *R. rubrum* (94) is controlled by DPNH through the activation of ribulose-5-phosphate kinase (EC 2.7.1.47). Further work must be done with these systems before the importance of the DPNH effect can be evaluated.

NATURE OF ENZYMES IN AMPHIBOLIC PATHWAYS

Having discussed the physiological aspects of control mechanisms so far, the important problem remaining is the nature of enzymes which serve as receptors of control signals. Two properties may be considered to characterize the regulatory enzymes sufficiently: (i) the presence of a distinct and relatively specific site on the enzyme which binds the regulatory ligand and (ii) the sigmoidal (or cooperative) velocity response given by most, if not all, of these enzymes to increasing concentration of substrate under certain conditions. In regard to the latter property, the allosteric enzymes of the amphibolic pathways can be divided (Fig. 5) into two clear-cut categories (Table 2): those which show modulator-independent cooperativity (MIC systems), i.e., which yield

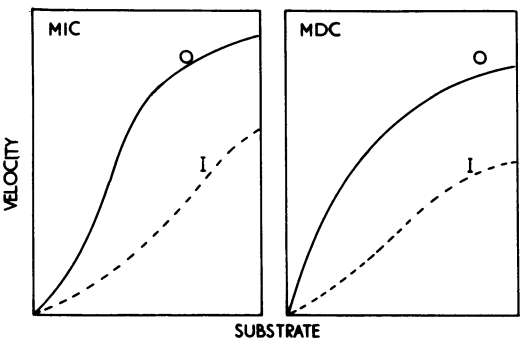

FIG. 5. *Typical plots of rate-concentration data obtained with MIC (modulator-independent cooperative) and MDC (modulator-dependent cooperative) systems in the absence (indicated by O) and presence (indicated by I) of inhibitors.*

TABLE 2. *Nature of allosteric enzymes in amphibolic pathways of E. coli*

Enzyme	Classification	Inhibitor	Activators	Target substrate	References
ADPG-pyrophosphorylase	MDC (?)	AMP	FDP, PEP, triose-P		90, 91
Citrate synthetase	MDC	DPNH, α-KG	K^+ (?)	OAA (DPNH), acetyl-CoA, (α-KG)[a]	131, 142
Glucose-6-phosphate dehydrogenase	MDC	DPNH	Spermidine	TPN	99
DPN-specific malate dehydrogenase	MDC	DPNH		Oxalacetate, malate	97
DPN-specific malic enzyme	MDC	CoA, ATP	Aspartate	Malate	98
TPN-specific malic enzyme	MDC	DPNH, TPNH oxalacetate, acetyl-CoA, 3′-5′-AMP	NH_3	Malate	103–105
PEP carboxylase	MIC	Aspartate, malate	Acetyl-CoA, FDP, GTP, CDP	PEP	10, 18, 100–102
PEP carboxykinase	MDC	DPNH		Oxalacetate	143
Phosphofructokinase	MIC	PEP	ADP, GDP	Fructose-6-phosphate	6, 8
Phosphotransacetylase	MDC	DPNH, ATP	Pyruvate	Acetyl phosphate	116
Pyruvate dehydrogenase	MIC (?)	Acetyl-CoA	AMP, GDP, PEP	Pyruvate	110
Pyruvate kinase (FDP activated)	MIC		FDP	PEP	72, 74

[a] See explanation in the text.

sigmoidal rate-concentration plots in the absence of inhibitors and activators, and those which yield sigmoidal initial velocity plots only in their presence [modulator dependent cooperativity (MDC)]. It is not necessary to confine this classification to the enzymes of the amphibolic pathways. With a few exceptions, all allosteric enzymes known so far in diverse pathways can be placed in one or the other of these categories. To this extent also, the discussion which follows is indeed applicable to all allosteric enzymes.

Although the physiological advantages accruing to an organism by possessing enzymes which give sigmoidal rate-concentration curves are obvious, the actual molecular mechanism underlying this behavior is certainly not known. Starting with the early proposals of Monod et al. (81) for the MIC systems, the trend is to analyze the kinetic behavior of allosteric enzymes in terms of subunit interactions, whether they involve simple isomerization or association-dissociation of the subunits (4, 29, 114). According to Monod's hypothesis, cooperativity is caused by the differential binding of substrates to two conformational states of the protein which are in equilibrium determined by a dimensionless constant, L. If this constant is such that most of the protein exists in a state which has very low affinity for the substrate (state T, or tight), cooperativity ensues because the substrate pulls the enzyme towards a state (R, or relaxed) which has much higher affinity for it. In this theory, for reasons of symmetry, hybrid states between T and R are considered almost nonexistent. Other hypotheses (4, 56), however, do not assume equilibrium between T and R states but consider that conformation of individual subunits can be changed by binding of ligand such that forms are possible where one subunit is in the R state and another in the T state.

As applied to the kinetic data, however, there is no reason a priori to consider sigmoidal velocity response as resulting exclusively from subunit interactions. A number of authors (107, 114, 141) have interpreted cooperativity of substrate with some enzymes of MIC category on the basis of the presence of two kinds of substrate binding sites on the enzyme surface, or even on the basis of the presence of alternate modes of binding of substrate molecules to single active sites (117). Much of this difference of opinion can be traced to the nature of techniques used in arriving at conclusions. Subunit isomerizations can only be detected

by physicochemical methods, such as equilibrium dialysis or stopped-flow techniques. Because varying degrees of nonhyperbolic character of rate-concentration plots can arise by changes in magnitudes of rate constants associated with various reactants, kinetic studies are least suited for deducing the nature of allosteric mechanisms with any degree of certainty. As an illustration, phosphofructokinase of *E. coli* (8) and muscle (76), both of which are MIC systems, behave kinetically in a way which is explicable on the basis of the Monod model. However, for the muscle enzyme at least, cooperativity of the substrate is not discernible in equilibrium ligand-binding studies (54), which perhaps means that part of the cooperativity observed in initial velocity studies, at the minimum, must arise from kinetic interactions, i.e., through modifications of certain critical rate constants (71, 114, 141). Similar uncertainties exist in the case of another glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) from yeast. Analysis of temperature-jump kinetics indicates (55) that for this enzyme cooperativity can be explained on the basis of Monod's model. However, using the formation of enzyme-substrate complex and rate of product release as criteria, another group of workers (15) consider cooperativity to be due to the presence of unproductive binding sites for the substrate on the enzyme surface.

To some extent, the same kinds of uncertainties of interpretation existing with MIC systems also apply to the MDC systems (71, 104, 143), but the interpretation is comparatively simpler with the latter category of enzymes. It may be recalled that the rate-concentration plots in MDC systems are always hyperbolic in the absence of inhibitors, but only become sigmoidal in their presence. This behavior necessarily means that the catalytic sites must all be equal and function independently of each other in the absence of the inhibitors. Very simply, the sigmoidality of the rate-concentration plots may then be explained on the basis of the isomerization of subunits (104) in the presence of the inhibitor. One can assume, for instance, that out of two final states of the subunits (R and T) possible, an enzyme constituted of identical subunits exists primarily in the R state (a state which has high affinity for substrate). The inhibitor binds at a specific regulatory site and converts the subunits to the T state which has low affinity for the substrate. With these assumptions, it can be readily realized (i) that without the inhibitor (since state T is almost nonexistent) the substrate will give hyperbolic rate-concentration plots because only the high affinity state is available; (ii) that in the presence of unsaturating concentrations of inhibitor (since the enzyme is now distributed into R and T states) the substrate will give a sigmoid response because it now binds with different affinities to the two states; (iii) that when the inhibitor is saturating and all of the enzyme is in the T state the substrate will again give a hyperbolic response, because it is now able to bind (albeit, with low affinity) only to the available T state; and (iv) that the inhibitor despite its structural dissimilarity will give competitive inhibition against substrate, because the former stabilizes the T state and the latter the R state and a push-pull situation will develop.

The actual experimental data obtained with MDC systems are compatible with this model only for the case of malic enzyme (104) and citrate synthetase (142), the inhibitors of which give competitive inhibition against at least one of the substrates. For malate dehydrogenase and PEP carboxykinase, however, the inhibition (97, 143) is noncompetitive. This fact raises the possibility that either cooperativity of the substrate arises in these cases due to kinetic interactions (i.e., changes of rate constants by the binding of the inhibitor) or the velocity constants for product release of R and T states are quite different.

TARGET SUBSTRATES

It is clear from what has been said above that in the present state of our knowledge only reasonable guesses can be made about the mode of action of allosteric enzymes. Indeed, in the above discussion the regulatory enzymes have been treated as if they had only one substrate, a situation which occurs only rarely. Most of the regulatory enzymes catalyze reactions in which two or more substrates participate, and this introduces a large number of complications in kinetic analyses; the greater the number of substrates and modulators, the greater becomes the possibility that any one single mechanism may not be able to explain the kinetic intricacies. Whatever the kinetic mechanism, however, the finding of greater physiological interest is that for most of the MIC and MDC systems of enzymes, it is generally only one of the two or more substrates which gives a sigmoidal velocity response. As an example, phosphofructokinase (MIC system) has two substrates, ATP and fructose-6-phosphate, and only the latter yields cooperative rate-concentration plots. Similarly, for malic enzyme (MDC system), only one of the substrates, malate, gives a sigmoidal velocity response in the presence of diverse inhibitors. Such a substrate may be termed a physiological target substrate because it is very likely that the occurrence of an allosteric control on the enzyme is directed towards the distribution of that particular substrate through the modulated pathway. Sigmoidality has the inherent ad-

vantage that the affinity (the reciprocal of Michaelis constant) of the substrate for the enzyme can be decreased (or increased) sharply over a narrow threshold range, and this characteristic makes possible a sensitive control system.

At first glance, the concept of a target substrate may seem trivial because a majority of bireactant allosteric enzymes utilize ATP (or ADP) or one of the coenzymes, DPN or TPN, as the second substrate. Since these nucleotides are shared by a large number of enzymes, common sense suggests that no useful purpose would be served by having these compounds as target substrates. It is physiologically meaningful always to have the nonnucleotide substrate as the target substrate, and this is also the actual experimental finding (Table 2). There are, however, a number of regulatory enzymes, both in amphibolic as well as other biosynthetic pathways, which do not utilize the nucleotides as the second substrate and in which the finding of a target substrate has considerable physiological significance. As an example, one may consider the case of citrate synthetase of *E. coli* (142; Wright and Sanwal, *unpublished data*) which has two substrates, acetyl-CoA and oxalacetate, and two allosteric inhibitors, DPNH and α-KG. Since citrate synthetase belongs to the MDC system, both substrates yield Michaelis-Menten kinetics in the absence of the inhibitors. With α-KG as inhibitor, the initial velocity plots with oxalacetate as a variable substrate remain hyperbolic (142) but acetyl-CoA plots become sigmoidal, whereas acetyl-CoA plots remain hyperbolic but oxalacetate plots become sigmoidal in the presence of DPNH. Physiologically speaking, acetyl-CoA is the target substrate for α-KG as oxalacetate is for DPNH; i.e., the inhibition by DPNH in the latter case perhaps primarily affects the distribution of oxalacetate through the modulated enzyme. Whereas both of the inhibitors will block formation of citrate, the concentration of oxalacetate will only rise *steeply* (however momentarily) in the presence of DPNH and that of acetyl-CoA will do so only in the presence of α-KG. Indeed, this sophisticated control device may be used in vivo to control efficiently the fate of certain compounds such as acetyl-CoA in the enteric bacteria. It is well known that in animals tricarboxylic acids determine the fate of acetyl-CoA by activating acetyl-CoA carboxylase (EC 6.4.1.2.), the first enzyme of fatty acid biosynthesis (9, 89). In *E. coli* however, no activators or inhibitors of this enzyme have been found (1), and it seems quite possible that here the distribution of acetyl-CoA is solely determined by feedback inhibition of citrate synthetase by α-KG. This inhibition would not only cut off the synthesis of α-KG but would also afford a steeply increasing supply of acetyl-CoA to the fatty acid synthesizing system.

CRITERIA FOR CONTROL

The implicit assumption that has been made in the discussion of controls of amphibolic pathways in bacteria is that the in vitro findings apply to conditions in vivo. This may not always be so, and to this extent the whole discussion may be considered conjectural until all the postulated controls have been demonstrated in situ in bacterial cells. Biochemical technology, however, has not progressed far enough to attempt this approach. Nevertheless, certain criteria must be available by which to judge whether inhibition or activation of an isolated enzyme in vitro plays the same role inside the cells. One such reasonable criterion is to show that the enzyme possesses an allosteric site for the modulator. This can be achieved by actual desensitization (80) of the enzyme to the effect of the modulator by physical or chemical treatments. A number of enzymes, however, are refractory to desensitization. If such enzymes belong to the MDC category, presence of an allosteric site can be clearly inferred from the kinetic data, because there is no conceivable way to generate sigmoidal rate-concentration plots of the substrate in the absence of an allosteric ligand-binding site.

There are, however, no compelling reasons to consider that only enzymes which carry regulatory sites are important in the control of intermediary metabolism. Interactions of two or more metabolites at the catalytic site of enzymes may contribute significantly to overall regulation of pathways. Atkinson (5) recently proposed that to a significant degree amphibolic pathways may be regulated by the energy charge (defined as ATP + 0.5 ADP/ATP + ADP + AMP) of the adenylate pools by interactions at the catalytic sites of enzymes and, furthermore, these interactions may be modulated in the case of regulatory enzymes by the binding of allosteric effectors. Although a considerable amount of indirect evidence supports this hypothesis, it would clearly be impossible at the present time to demonstrate these events in the cells.

Despite the difficulties of demonstrating controls in vivo, approaches are still available in bacterial systems which could profitably be used for this purpose. In *E. coli* for instance, mutants are available (58) which have a thermolabile adenylate kinase. It should be possible in such strains to measure steady-state concentrations of metabolites which are suspected of being regulated by energy-linked controls (such as FDP, acetyl-CoA, pyruvate, etc.) under conditions in which adenylate kinase is fully active and inactive. Sim-

ilarly, cold-sensitive mutants are available (86) and others could possibly be made which show altered control of specific regulatory circuits. In fortunate circumstances, it may even be possible to select for regulatory mutants not showing any conditional phenotype. Cattanéo et al. (13) and Govons et al. (35) recently selected mutants of *E. coli* which accumulate glycogen (judged by the intensity of iodine staining), and this has been shown to result from altered regulatory properties of ADP-glucose pyrophosphorylase which in the mutant shows a much lower affinity for the inhibitor (AMP) and much higher affinity for the activator (FDP) compared to the enzyme from wild-type *E. coli*. Yet another approach with enteric bacteria, particularly *E. coli*, consists in the treatment of the cells with chelating agents such as ethylenediaminetetraacetic acid (62, 63). In this way, cells can be made permeable to a number of phosphorylated compounds (ATP, ADP, etc.) and control properties of enzymes can be measured in situ. This approach has been used by Liersch and Preiss (67) to assay ADP-glucose pyrophosphorylase and to study its control characteristics in vivo.

CONCLUSIONS

The discussions of controls in amphibolic pathways in bacteria have so far been a mixture of a number of facts and a large amount of speculation. Although the speculations themselves will have to be subjected in the future to the rigorous scrutiny of experimentation and may or may not hold ground, the factual information itself raises the question whether any heuristic generalizations are at all possible from the study of the controls of one organism or a group of organisms which may be applicable to other groups of organisms. The answer seems to be that the "unity of biochemistry" concept which was so useful for the study of intermediary metabolism per se in diverse organisms may not hold so far as coordination and control are concerned. A case in point, for instance, is the regulation of citrate synthetase and the consequent possibilities of partition of acetyl-CoA into the citric acid cycle or the pathway of fatty acid biosynthesis. In animals (47), yeast (87), and *B. subtilis* (25), this enzyme is inhibited by ATP. In *E. coli*, on the other hand, citrate synthetase is inhibited by DPNH and α-KG. Still, in another group of aerobic gram-positive bacteria, the enzyme is not regulated by ATP or DPNH (134), so that these organisms will possibly be found to have quite different control mechanisms for restricting the flow of acetyl-CoA into different channels. It seems that control mechanisms have evolved by selection to suit the needs of a particular organism growing in its habitual milieu. Such being the case, diversity rather than a narrow unity would be the desirable feature of control.

One may conclude this essay by pointing out the problems that remain to be solved in the area of control of amphibolic pathways in bacteria. One obvious problem, albeit not too exciting, is the comparative biochemistry of the feedback systems in groups or tribes of bacteria. These may yield valuable clues to taxonomic relationships among different forms (134). Since most of the contemporary knowledge about controls is based on *E. coli*, one of the attempts of this review has been to predict possible control points in other bacteria from an analysis of existing knowledge. Study of these enzymes may unearth systems which have properties amenable to detailed physicochemical analysis. It is the study of this fundamental area that alone will lead to advances in our understanding of allosteric phenomena. To date, essentially only three enzymes, aspartate transcarbamylase from *E. coli* (32), glutamine synthetase (EC 6.3.1.2) from *E. coli* (140), and bovine glutamate dehydrogenase (30), are known which have the prerequisites, namely, ease of isolation in large quantities, susceptibility to desensitization, and possibilities for enzymatic or chemical modifications of amino acid residues, for further work to be profitable.

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